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Purification of recombinant HBc antigen expressed in *Escherichia coli* and *Pichia pastoris*: comparison of size-exclusion chromatography and ultracentrifugation

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Abstract

Hepatitis B virus core protein (HBc) is an important serology marker of hepatitis B infection and patient follow-up. It is an M_r 21 000 protein, which has the intrinsic capacity to self-assemble as a capsid-like particle. The hepatitis B core protein has been expressed in *Escherichia coli* and *Pichia pastoris* (three different constructions) in order to select a HBc recombinant antigen suitable for serodiagnosis requirements with a cost effective downstream strategy. The expression and purification of the different forms of recombinant HBc have been described. For the last step, ultracentrifugation and size-exclusion chromatography were compared. The morphology of these capsids was observed using an electron microscope. Our data shows that HBc antigen is produced in large quantities in *E. coli* but some contaminants remained which were associated with the *E. coli* HBc protein after ultracentrifugation or size-exclusion chromatography. The ultracentrifugation enables a higher purity of HBc antigen to be obtained than size-exclusion chromatography but the latter enables a higher recovery rate. *P. pastoris* enables the expression and extraction of a highly purified HBc antigen suitable for diagnostic purposes.

Keywords: Purification; Hepatitis B virus core protein

1. Introduction

Hepatitis B, which is an enveloped DNA virus of the *Hepadnaviridae* family, remains one of the most widespread lethal infectious diseases worldwide. Chronic infection can lead to cirrhosis and hepatocellular carcinoma. Despite vaccination programs and screening of blood donors, HBV is still a major public healthcare problem with around 400 million chronic HBV carriers in the world. For initial diagnosis, patient monitoring and differentiation between acute and chronic infection, there is a need for specific serological markers (among these, detection of IgM and IgG anti-HBc Ag signal either acute or chronic infection).

The virus contains an outer lipoprotein envelop composed by the surface antigen (HBs antigen) and an inner nucleocapsid containing a double-stranded DNA genome and viral proteins. The nucleocapsid is composed of a molecular mass (M_r) 21 000 protein of 183 amino acid residues named core antigen (HBc antigen), which self-assembles during viral replication in liver cells, to form a regular 20-hedron structure.

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Due to the lack of an efficient in vitro propagation system for HBV and the difficulty of isolating significant quantities of virus from infected human liver, HBc antigen (Ag) was first efficiently expressed in *Escherichia coli* [1–3], with intracytoplasmic particle formation. Indeed, it has been shown that recombinant HBc is able to dimerize and self-assemble in particles.

Recent studies with HBc expressed from *E. coli* [4] showed that the icosaehedral shell was composed of 180 or 240 subunits. The C-terminal region (150–183) of the HBc protein, a very basic protamine like domain, is not involved in capsid assembly [5] but is reported to interact with nucleic acid [6]. The capsid structure has been recently ellucidated by crystallography and cryoelectromicroscopy, and underscore the dimer clustering producing spikes on the surface of the shell [7–9].

The expression of foreign genes in different host systems has been widely explored over the past 2 decades, and it is well known that the choice of expression system (host, plasmid), is a key point for successful production of a correctly folded protein. Previous studies have reported the expression of full length or truncated sequences of HBc protein mostly in *E. coli* under an inducible promoter, but also in other hosts such as yeasts and insects cells [10]. Usually, the purification processes are based on the physical properties of the HBc core particles, i.e., size and density. Moreover, sedimentation on sucrose gradient by ultracentrifugation is the method of choice largely reported. However, size-exclusion chromatography has also been used. For over 40 years, gel filtration supports have been designed for the separation of a large varieties of macromolecules according to their size and ability to penetrate a sieve-like structure. Virologists were also quick to use and apply this principle for virus particle purification [11]. More recently, another strategy has been designed using fusion tails. This approach promotes efficient recovery and purification from crude cell extracts [12] and was recently implemented with an expressed tagged HBc histidine purified by metal chelate affinity chromatography [13].

In an attempt to produce an HBc recombinant antigen of use for hepatitis serodiagnosis, we evaluated different expression systems to express a well folded and polymerized antigen and developed a cost effective and easily scaled up purification process.

In this paper, we have compared the expression and purification processes of HBc antigen in *E. coli* and *Pichia pastoris*. Two constructions were designed to produce the recombinant HBc protein in the *E. coli* bacterial cytoplasm: the first constitutively expressed HBc alone, and the second expressed HBc fused to a C-terminal hexa-histidine tag, under the control of the tac promoter. Finally, the HBc cloned in *P. pastoris* is expressed under the control of the promoter alcohol oxidase gene.

The efficiency of the three extractions protocols applied have been evaluated, and have been followed by comparison of the polishing steps: ultracentrifugation on sucrose gradient and size-exclusion chromatography. The respective purified fractions of HBc particles have been checked by analytical size-exclusion chromatography and electron microscopy imaging. We have shown that ultracentrifugation provided better purity than size-exclusion chromatography, but the latter enabled a higher recovery rate of the HBc antigen.

2. Experimental

2.1. Recombinant constructions, cultures and extractions

The different constructions and their respective culture and extraction protocols are described briefly below.

2.1.1. E. coli E3CB

2.1.1.1. Culture. The coding DNA for HBV core protein (amino acids 2–185) was isolated by polymerase chain reaction (PCR) from a HBV infected patient and subcloned under the tac promoter in pPROCK-C (Clontech, Palo Alto, CA, USA) vector and used to transform *E. coli* JM109.

Bacteria were grown in a 5-l shaker flasks (280 rpm, 37°C) in 1 l Luria Broth (Difco, Detroit, MI, USA) in the presence of Ampicillin (Roche Boehringer, Mannheim, Germany) at 100 μg/ml, until reaching an absorbance at 600 nm ($A_{600\ nm}$) of 2.0. Bacteria were harvested by centrifugation at 6000 *g* for 30 min at +4°C, and the pellets were processed immediately or stored at −80°C.

2.1.1.2. Extraction. The bacterial pellet was resuspended in 20 ml of extraction buffer [30 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA, 20% saccharose, 0.1 ml/100 ml Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)] for 5 min at room temperature. Then, the suspension was centrifuged at 10 000 *g* for 30 min at room temperature. The pellet was resuspended with cold water for 10 min and centrifuged again at 5000 *g* for 15 min at +4°C. The pellet was then suspended in lysis buffer (50 m*M* Tris–HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA, 1 mg/ml lysozyme, 1 m*M* PMSF), and stirred overnight at +4°C. Lysis was achieved by four successive freezing in liquid nitrogen and thawing stages. DNase I (10 μl/ml, Roche Molecular Biochemical, Mannheim, Germany) was added and incubated at room temperature until the suspension was fluid. The suspension was then centrifuged at 11 000 *g* for 30 min. The supernatant, containing the HBc protein was diafiltered on Miniultrasette M_r 300 000 cut-off membrane (Pall Filtron, Northborough, MA, USA) with 10 m*M* sodium phosphate, 140 m*M* NaCl, pH 7.2 (phosphate-buffered saline, PBS) until the protein concentration in the ultrafiltrate decreased. The diafiltrate was stored at 4°C before the final purification step.

2.1.2. E. coli pOL032

2.1.2.1. Culture. The same DNA sequence reported above was cloned, in fusion with a 6 histine tag at the C-terminal end, in the pMR vector [14], which was used to transform *E. coli* BL21.

This strain was grown in shaker flasks overnight (280 rpm, +37°C) in 2YT medium (Difco), containing 2% glucose and 100 μg/ml Ampicillin. On the following morning, 20 ml of the culture was inoculated into a 1-l medium flask and growth continued while shaking until an $A_{600\ nm}$ 0.8 was reached. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Roche Molecular Biochemicals) to a final concentration of 1 m*M*. After 3 h growth, the cells were harvested by centrifugation at 6000 *g* for 20 min at +4°C.

2.1.2.2. Extraction. The pellet corresponding to 1 l of culture was resuspended in 20 ml lysis buffer (50 m*M* NaH_2PO_4, 6 m*M* imidazole, 8 m*M* deionised urea, 5% sucrose, 1 m*M* PMSF). The cells were lysed by sonication (6×30 s) at +4°C; after 30 min incubation at +4°C, the lysate was centrifuged at 14 000 *g* for 30 min. Ni^{2+} chelate adsorbent (Ni-NTA Agarose; Qiagen, Hilden, Germany) was equilibrated in lysis buffer containing 0.5 *M* NaCl to avoid ionic interactions. The soluble bacterial extract was then mixed with 4 ml of the gel under rotating agitation overnight at +4°C. The gel was packed in a column for the washing steps with the same buffer. The best elution conditions of the fusion proteins were obtained with lysis buffer to which 0.5 m*M* imidazole was added. The denatured fusion protein in the elution fraction was dialysed (ratio 1:100) three times successively, against a renaturation buffer 100 m*M* Tris–HCl, pH 8 containing 0.5 *M* arginine, 0.2 *M* reduced glutathione, 2 m*M* oxidised glutathione, 2 m*M* EDTA while stirring for 1 day at 4°C.

2.1.3. P. pastoris yBW063

The DNA sequence coding for HBV core protein was cloned in the pPIC3.5K vector (InVitrogen, Groningen, The Netherlands). The resulting construction was used to transform the GS115 *P. pastoris* strain.

2.1.3.1. Culture. The recombinant yeast was grown in shaker flasks with 100 ml ''BMGY'' medium (1% yeast extract, 2% peptone, 100 m*M* potassium phosphate, pH 6, 1.34% yeast nitrogen base, $4\cdot10^{-5}$ *M* biotin, 1% glycerol) at 30°C until the $A_{600\ nm}$ reached 6. The cells were harvested by centrifugation (1500 *g*, 10 min) and resuspended in 1 l ''BMMY'' (1% yeast extract, 2% peptone, 100 m*M* potassium phosphate, pH 6, 1.34% yeast nitrogen base, $4\cdot10^{-5}$ *M* biotin, 0.5% methanol) medium to obtain an $A_{600\ nm}$ of approximately 1 and incubated for 4 days with 0.5% methanol supplement every 24 h to induce expression. The cells were harvested by centrifugation at 4000 *g* for 30 min at 4°C.

2.1.3.2. Extraction. The pellet was resuspended into cold lysis buffer (10 m*M* NaH_2PO_4, 1 m*M* EDTA, 5% glycerol, 1 m*M* PMSF, pH 7.4) at 1 g/ml (wet mass). The cells were disrupted in a bead beater (Biospec Products, Bartlesville, OK, USA) using glass beads of 0.5 mm diameter, during 5×1 min at 4°C. The homogenate was centrifuged twice: 30 min

at 3000 *g*, and then 30 min at 10 000 *g* at 4°C. The supernatant was collected and subjected overnight to ammonium sulfate precipitation, 40% saturation, at 4°C. The precipitated fraction was isolated by centrifugation at 18 000 *g*, for 30 min and then dissolved in NTE buffer (100 m*M* NaCl, 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.6). The soluble fraction was treated by heating at 65°C for 1 h. Contaminants were removed by centrifugation 10 000 *g* for 30 min and the supernatant containing the HBc protein was stored at 4°C.

The global extraction and purification schedule is summarised in Table 1.

2.2. Purification and analysis

2.2.1. Purification: preparative and analytical size-exclusion chromatography

For analytical size-exclusion chromatography, a Superdex 200HR column (300×10 mm; Pharmacia, Uppsala, Sweden) was used on a 1050 high-performance liquid chromatography (HPLC) system (Hewlett-Packard, Palo Alto, CA, USA) with a diode array detector. For preparative experiments, a Superdex 200 Prep Grade (600×16 mm) was used on a fast protein liquid chromatography (FPLC) system (Pharmacia). For each column, a calibration curve was established by using a protein calibration kit (ref. 17-0442-01, Pharmacia).

100 m*M* disodium phosphate, 150 m*M* NaCl, pH 8.0 was used as an eluent at the following linear flow-rate: 0.35 ml/min (preparative chromatography) and 0.5 ml/min (analytical chromatography). Samples corresponding to 10–20 mg of materials (5 ml for preparative chromatography) were clarified by centrifugation prior to injection on the column. A 0.05-ml volume was injected for analytical chromatography.

2.2.2. Sucrose gradient ultracentrifugation

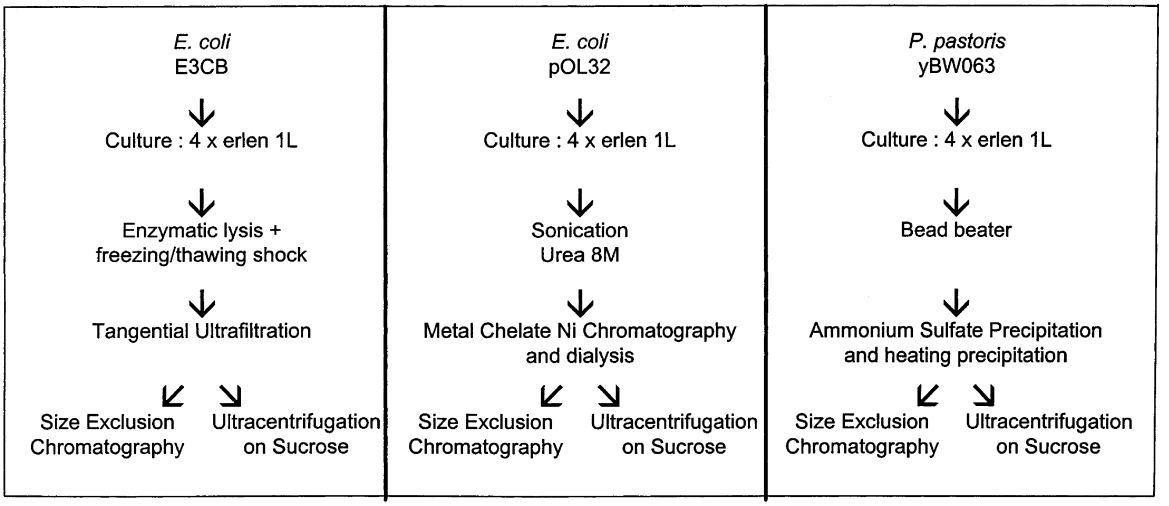
In order to purify the HBc core particles, the prepurified extract of *E. coli* E3CB, *E. coli* pOL032 and *P. pastoris* yBW063 were subjected to sucrose discontinuous gradient centrifugation.

SW28 polyallomer tubes (Beckman Coulter, Chaska, MN, USA) were filled successively with 2 ml of decreasing sucrose solution (60%, 50%, 40%, 20%). Then 2 ml of prepurified extracts, corresponding to 5–10 mg of materials was layered at the top of each tube. Centrifugation was performed at 112 000 *g*, at 4°C for 16 h. The gradients were drained by pipetting 1-ml fractions and subjected to protein analysis and enzyme immunoassay (EIA) testing.

2.2.3. Immunoassay

A direct immunoassay was performed to detect all

Table 1
Experimental – lysis protocol and first extraction steps applied to *E. coli* E3CB, *E. coli* pOL032 and *P. pastoris* yBW063



HBc antigen forms, preferentially to a sandwich immunoassay which is rather used to detect particles. The EIA plate was coated at room temperature overnight with 100 μl/well with dilutions of fractions of interest in 50 m*M* sodium carbonate buffer, pH 9.6. After three washes with PBS–0.05% Tween 20, the plate was blocked with 5% bovine serum albumin (Sigma, St. Louis, MO, USA) in PBS (200 μl per well, 1 h at 37°C). Then the plate was incubated with 100 μl/well of monoclonal antibody anti-HBc alkaline phosphatase conjugated (BioMérieux) for 1 h at 37°C. Then after three washes, enzymatic reaction was developed with 100 μl of 0.2 *M* Tris buffer, pH 8 containing 1 mg/ml *para*-nitrophenyl phosphate (pNPP; Sigma). After 15 min, the reaction was stopped by the addition of 100 μl of 1 *M* sodium hydroxide and absorbance was measured at 405 nm.

2.2.4. Analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis

Analytical SDS–PAGE on a 15% T, 3.3% C (% T=g acrylamide+g Bis)/100 ml solution; %C=g Bis/% T) gel, 1.5 mm thick was performed using Laemmli's method with Miniprotean II (BioRad, Hercules, CA, USA). Proteins were denatured in SDS sample buffer (2%, w/v, SDS, 62.5 m*M* Tris–HCl, pH 6.8, 5%, v/v, mercaptoethanol, 10%, v/v, glycerol and 0.0125% w/v Bromophenol Blue). A 35-μl volume was loaded per sample. After electrophoresis, the gel was stained with Coomassie blue and dried or electrotransferred for 45 min at 150 V, 300 mA, to an Immobilon-P polyvinylidone fluoride membrane (Millipore, Bedford, MA, USA). The membrane was incubated with blocking reagent (5% milk in PBS) at room temperature for 1 h and subsequently processed for 1 h with monoclonal anticore HBc antibody (BioMérieux) diluted 1:2000 in a solution of 10 m*M* Tris–HCl (pH 8), 150 m*M* NaCl and 0.05% Tween 20. After washing out the primary antibody, the membrane was processed with an anti-mouse phosphatase alkaline conjugated immunoglobulin (Jackson ImmunoResearch, West Grove, PA, USA). Then bands reacting with antibodies were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma).

2.2.5. Electron microscopy

The HBc purified fractions were observed under an electron microscope. Samples (5 μl) were placed on 200 mesh copper grids coated with collodion–carbon film and air dried. Grids were contrasted with an 2% aqueous solution of phosphotungstic acid and examined in a Philips CM120 transmission electron microscope (acceleration voltage: 80 kV).

2.2.6. Protein determination

Protein level of the different fractions is evaluated by Bradford assay (BioRad).

3. Results

3.1. Lysis and HBc extraction

The biomasses obtained from a 4 l culture of *E. coli* E3CB and pOL032 were, respectively, 42.5 g and 30.8 g, and 92 g for *P. pastoris*. These values are consistent with data reported for *E. coli* and *P. pastoris* strains and result from an 8-h culture with doubling times of 20 min. The yeast *P. pastoris* yielded a higher biomass but required a 4-day culture under methanol induction to achieve this level.

The results of the first extraction step of HBc fractions from the three strains are presented in Table 2. For each strain, specific extraction protocol was followed as described in Experimental (Table 1), and the comparison of the results was not possible in extenso.

Nevertheless, we can observe for the *E. coli* strains the impact of the lysis method on the total protein content in all extracts.

When considering the protein extracted per g biomass wet mass, the pOL032 lysis supernatant yields 50.3 mg protein against 9.8 mg with the E3CB lysis supernatant.

The lysozyme and freezing–thawing performed with *E. coli* E3CB released a fivefold less protein than the ultrasonic and urea treatment performed with *E. coli* pOL032.

For the *P. pastoris* strain, despite a higher biomass, the cell lysis using the bead beater, gave a rather low level of total protein extracted per g biomass wet mass: 4.4 mg. This could be due to an incomplete lysis.

Table 2
Extraction results for the three HBc constructions

Steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	mg Protein extracted/g biomass	Yield (%)
E. coli E3CB (wet mass for a 4-l culture: 42.5 g)					
Lysis supernatant	126	3.3	416	9.8	100
Ultrafiltrated fraction	550	0.05	25.8	0.6	
Diafiltrated fraction	18	20	360	8.5	86.5
E. coli pOL032 (wet mass for a 4-l culture: 30.8 g)					
Lysis supernatant	125	12.4	1550	50.3	100
Imidazole fraction	44	3.23	142	4.6	9.2
Dialysed fraction	43	1.95	83.8	2.7	5.4
Concentrated fraction	16	5.4	86.4	2.8	5.6
P. pastoris yBW063 (wet mass for a 4-l culture: 92 g)					
Lysis supernatant	135	3	405	4.4	100
$(NH_4)_2SO_4$ supernatant	260	4	1040		
$(NH_4)_2SO_4$ pellet	17	1.8	30.6	0.33	7.5
65°C supernatant (HBc)	8.3	1.4	11.6	0.12	2.8

The extraction of HBc from *E. coli* E3CB by tangential flow concentration and diafiltration on the M_r 300 000 cut-off membrane, enables a weak protein reduction of 14%, but was inefficient to remove the contaminant proteins. For the *E. coli* pOL032 extract, the HBc protein expressed with the C-terminal His tag allowed purification on Ni-NTA agarose gel. The majority of the contaminant proteins were eliminated in the flowthrough process and elution using imidazole allowed the recovery of a 86 mg protein fraction which corresponded to an 18-fold protein decrease after renaturation dialysis and a global yield of 5.6%.

For the yBW063 yeast extract, a double precipitation protocol was performed. The first 40% ammonium sulfate leads to the precipitation of HBc protein and some contaminant material. The protein content was 405 mg before precipitation and was reduced to 30 mg after precipitation. The resolubilised pellet was heated for 1 h at 65°C which induces the precipitation of contaminants while the HBc protein remains soluble. From these results we deduced that HBc represents less than 2.8% of the total protein in the initial yeast extract.

3.2. HBc purification steps

Samples of these extracts were subjected to two purification steps: size-exclusion chromatography and ultracentrifugation.

The chromatogram in Fig. 1 demonstrates the resolving power of Superdex 200 to separate high-molecular-mass forms from low-molecular-mass components for the three HBc protein extracts. The pOL032 HBc antigen was eluted at the column void volume. For E3CB and yBW063, the peaks are eluted closed the void volume. The UV 280 nm profile tends to reflect the heterogeneity of each extract.

As determined by the enzyme-linked immunosorbent assay (ELISA) direct technique with the monoclonal anti-HBc antibody, the HBc immunoreactivity is concentrated in the first peak (Fig. 1). The other eluted fractions did not show high reactivity. These results prove the presence of high-molecular-mass forms of HBc protein. Moreover, these reactive fractions assayed by SDS–PAGE under reducing conditions showed the presence of an M_r 22 000 HBc Ag monomer band.

The same extracts were subjected to a sedimentation on a sucrose discontinuous gradient by ultracentrifugation. From the top to the bottom, fractions of 1 ml were sampled and then assayed using the EIA direct technique. The results are presented in Fig. 2 for the three extracts. Significant EIA responses are obtained in the 40–50% sucrose band. A slight difference can be observed for the highest response between each HBc particule extract.

At the bottom of the tube, some HBc reactive materials were pelleted.

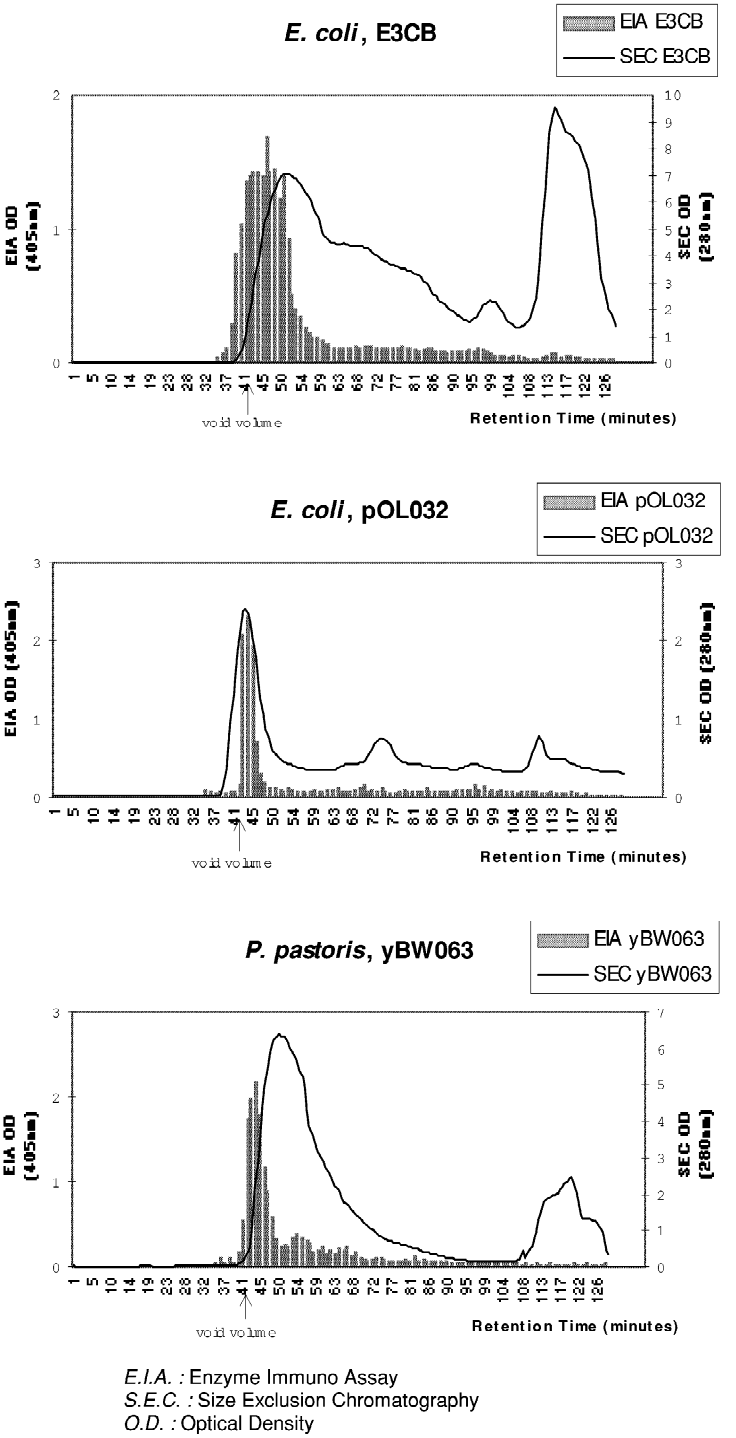


Fig. 1. Purification of the three HBc extracts by size-exclusion chromatography on Superdex 200 Prep Grade. Fractions (2 ml) were collected and assayed by direct EIA with an anti-HBc monoclonal antibody. The continuous curve corresponds to the $UV_{280\ nm}$ profile and the grey histogram represents the EIA *A* values. The column void volume determined with Blue Dextran is 42 min retention time.

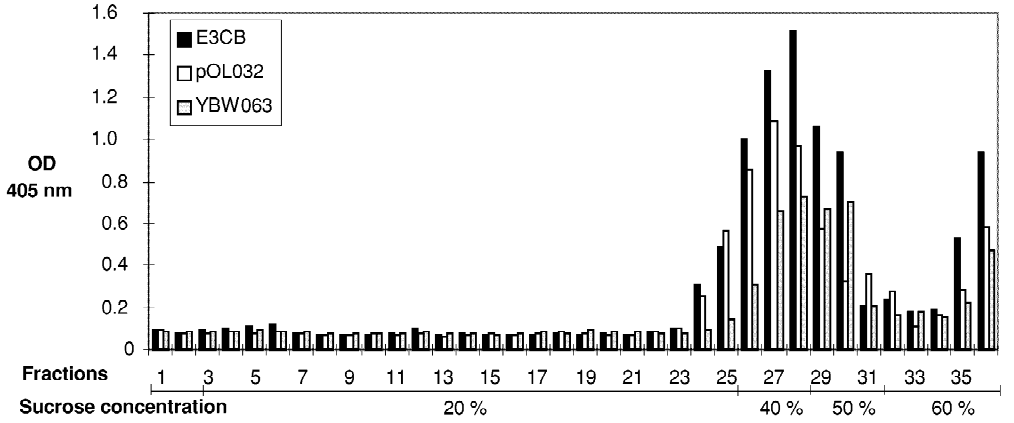


Fig. 2. Purification of the three HBc purified extracts by ultracentrifugation on sucrose density. Fractions (1 ml) were drained and assayed by direct EIA with an anti-HBc monoclonal antibody. The histogram represents the EIA *A* values.

Electron microscopic observations were performed on the 40% sucrose fractions and on the three chromatographic peaks of each HBc extract. The micrographs are presented in Fig. 3.

In the three cases, HBc capsid particles are visible and at a homogeneous size. For HBc E3CB, pOL032 and yBW063 the average diameters are, respectively, 44, 30.5 and 34 nm (determined on 50 particles), against 27 nm as previously described [15]. The HBc particles isolated by sucrose sedimentation appear more aggregated than the homologous particles isolated by size-exclusion chromatography.

The purity of the HBc core fractions have been checked by analytical size-exclusion chromatography (Fig. 4) on Superdex 200 HR and by SDS–PAGE Coomassie Blue stained electrophoresis (Fig. 5).

The chromatographic profile of each HBc core showed a major peak at the exclusion volume but with few low molecular mass absorbing materials ($<M_r$ 20 000) for the HBc fractions isolated by sucrose sedimentation.

The gel electrophoresis under reducing conditions showed different pattern between the two purification methods (Fig. 5). The HBc fractions from size-exclusion chromatography, lanes 2, 4 and 6 presented an M_r 22 000 distinct band which correspond to monomeric forms of HBc but also some background for *E. coli* E3CB and pOL032 extracts entrapped with HBc particles. However, the HBc fractions from sucrose sedimentation, lanes 1, 3 and 5, revealed the monomeric HBc polypeptides at M_r 22 000.

The final yield and productivity results obtained for the three constructions by the size-exclusion chromatography and sucrose sedimentation are given in Table 3 and represent the global values for the whole extraction and purification process. The best purified HBc particles were obtained from *P. pastoris* by ultracentrifugation but with the lower yield 1.6%. The size-exclusion chromatography purification led to higher recovery (8.5–24%) than ultracentrifugation (1.6–4.5%).

4. Discussion

The expression and purification of the same sequence in different hosts is not easy to compare, and even less when the purification of the expression product is carried out in different ways for each host. This approach is however necessary, particularly in the case of an industrial project where not only the product quality, i.e., activity, folding of the recombinant protein, but also the whole process, i.e., practicability and robustness must be considered. All these factors, including the global purity and yield, will have an impact on the value of the raw material. Our results lead us to discuss about the respective efficiency of each lysis protocol applied, and the first extraction step. Finally, we will compare ultracentrif-

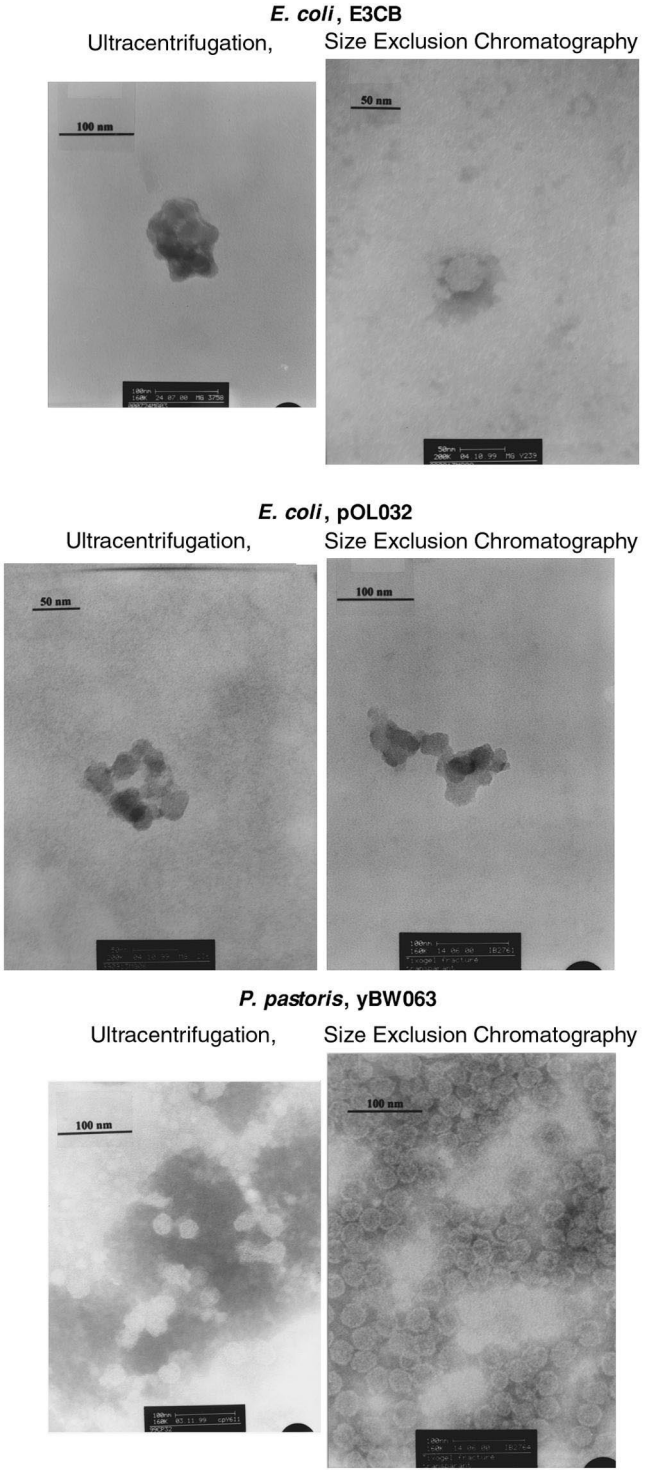


Fig. 3. Electron micrographs of purified HBc particle by ultracentrifugation or size-exclusion chromatography. Samples were negatively stained with 2% phosphotungstic acid. Electron micrographs were performed on a Philips CM120 electron microscope (80 kV). The scale is indicated on each picture.

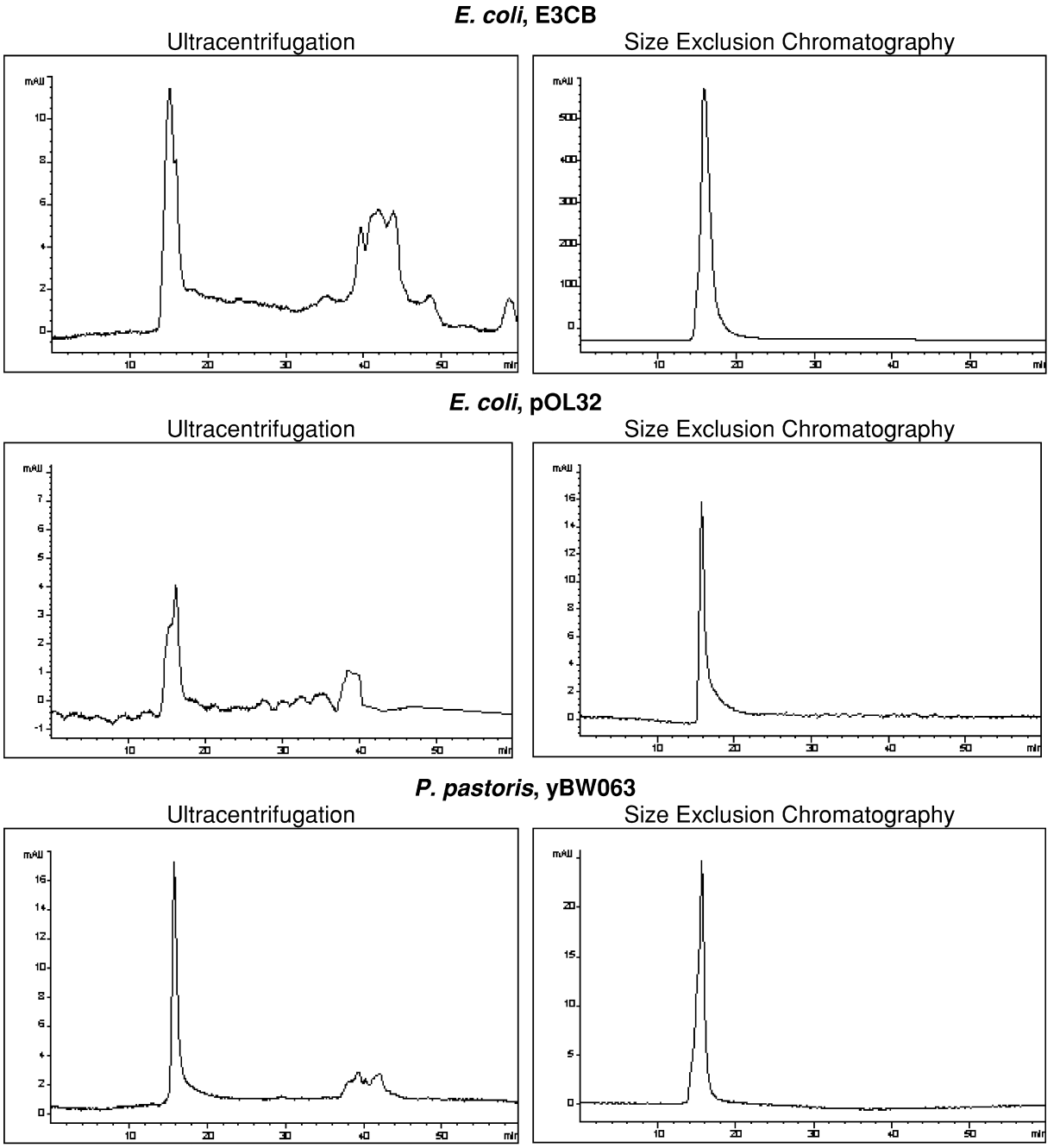


Fig. 4. Analytical size-exclusion chromatography profiles of the HBc fractions purified from size-exclusion chromatography and sucrose sedimentation. A 0.05-ml volume of each fraction was injected on a Superdex 200HR column.

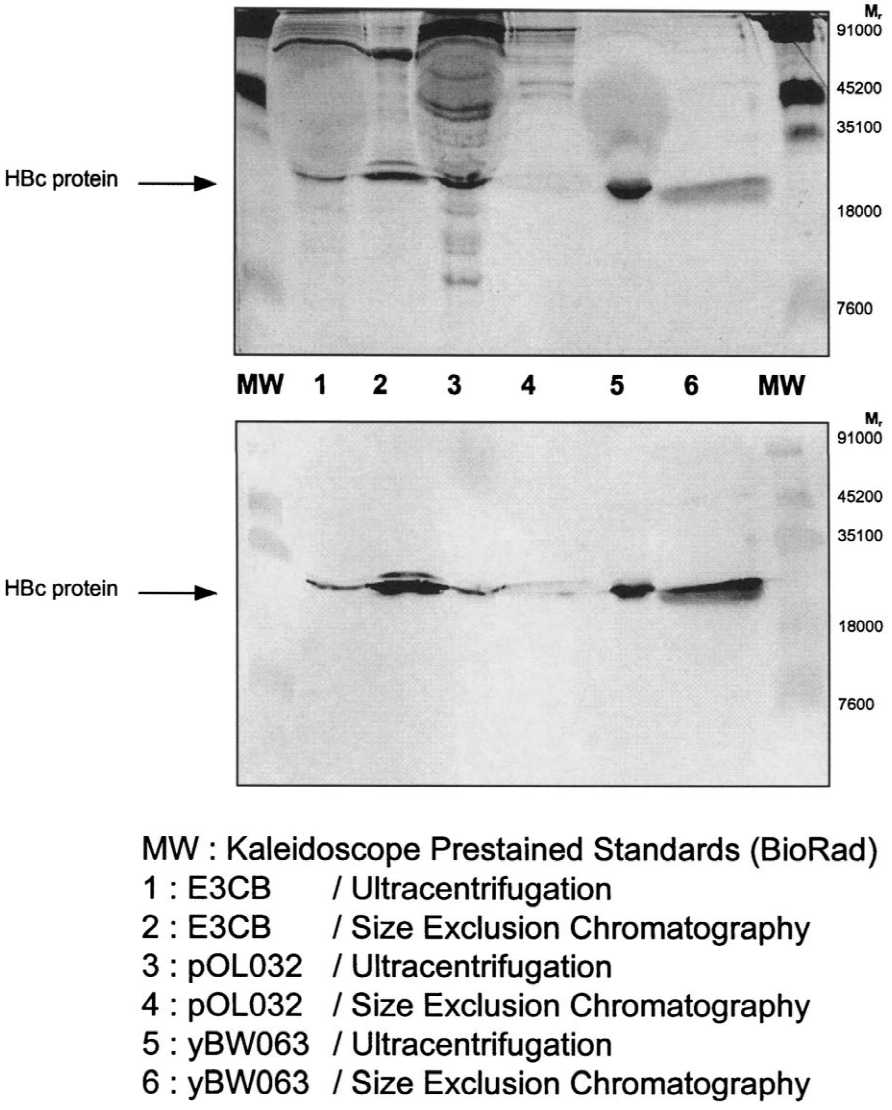


Fig. 5. SDS–PAGE and Western Blotting of HBc protein expressed in *E. coli* E3CB, *E. coli* pOL032 and *P. pastoris* yBW063 purified by size-exclusion chromatography and ultracentrifugation. The transferred membranes were probed with an anti-HBc monoclonal antibody. The HBc monomeric form is indicated by an arrow.

ugation and size-exclusion chromatography as final polishing steps.

The recombinant HBc core proteins have been expressed using a variety of cell systems, mostly bacteria (*E. coli*), but also in yeast [16,17], *Xenopus* oocysts [18], insect cells [19] and mammalian cells [20]. For this project, HBV DNA encoding the open reading frame for the core protein was cloned into vectors used to transform two *E. coli* and one *P. pastoris* strains. The transformed strains were analysed for their ability to produce the recombinant HBc protein, and for its ability to polymerise HBc protein into particles. The formation of HBc core particles was examined by electron microscope imaging.

The most current lysis protocol for transformed *E.*

Table 3
Comparison of HBc extract purification by size-exclusion chromatography and ultracentrifugation[a]

		Size-exclusion chromatography	Ultracentrifugation
E3CB	Total protein yield (%)	24	2.37
(*E. coli*)	Productivity (mg/g of biomass)	2.050	0.201
pOL32	Total protein yield (%)	15	4.49
(*E. coli*)	Productivity (mg/g of biomass)	0.422	0.126
yBW063	Total protein yield (%)	8.57	1.63
(*P. pastoris*)	Productivity (mg/g of biomass)	0.011	0.002

[a] These data represent the global yield and productivity of the whole three processes.

coli cells, freezing–thawing with lyzozyme and DNAse treatment was reported for HBc expression [21,4,7]. Some authors have added an additional sonication step to improve lysis [5,13]. French Press and sonication were also successively employed [22]. After clarification by centrifugation, the supernatant is the starting material for further purification steps. From our results, the difference between the enzymatic lysis and freezing–thawing performed on the *E. coli* E3CB cells can be seen with a protein content of 9.8 mg/g biomass wet mass, as well as the more drastic protocol applied to the *E. coli* pOL032 cells by sonication in presence of 8 *M* urea. The lysis is almost complete and releases about fivefold more soluble proteins than the previous protocol. As regards the expression in *P. pastoris*, it is well known that the yeast cell wall is thick and very resistant. The recommended lysis technique, by using a glass bead beater, gives a rather low level protein content (4.4 mg/g biomass wet mass), even with longer and repeated shaking time. It can now be observed that the protocols applied to the *E. coli* E3CB cells and *P. pastoris* are not denaturing and not reducing and allow the HBc core polypeptide to remain as a particle in favourable polymerising conditions. On the contrary, the denaturing and reducing conditions used with the *E. coli* pOL032 cells, lead to a solubilization of core polypeptide, and therefore, orient the first extraction step towards a metal affinity chromatography using the fused C-terminal histidine tag.

Most of the authors who have investigated HBc core particles have chosen to isolate HBc core particles by using the physical properties of the particles, i.e., size and density. This explains why ultracentrifugation has been the dedicated technique to isolate the HBc core particles, as a pellet [22] or on sucrose gradient or cushion [10,13,23].

An alternative technique, size-exclusion chromatography has also been used for the same purpose. Sephadex G50 was first used as gel filtration support [2] to isolate HBc antigen directly from a crude supernatant. Other supports (Sephacryl S 300, Sepharose CL4B) were also used as a second purification step. Some authors have described an extraction protocol using precipitation with 30% ammonium sulfate [4,7,5]. This is useful to eliminate contaminants from host cells, and also allows a volume reduction. The solubilized pellet was then desalted and purified from coprecipitated contaminants by size-exclusion chromatography on Sepharose CL4B with 0.1% Triton X-100 [22,4,7].

Two recent purification approaches have been described. The HBc core resistance [24] have been used to perform a 1-h heat treatment at 65°C to enable precipitation of contaminants remaining from a first ammonium sulfate precipitation [25]. A second approach [13] was used: the HBc core protein was expressed in *E. coli* with a fused C-terminal histidine tag, but the first purification step was devoted to HBc core particle extraction from crude supernatant by ultracentrifugation. Then, the HBc core particles were denatured by addition of 2 *M* urea and mercaptoethanol, and the HBc polypeptides were adsorbed on Ni-NTA agarose gel under the same conditions. After washing out of contaminants, the imidazole eluted fraction was then dialysed to enable polymerisation of HBc particles. Some authors also performed an additional polishing step by using ultracentrifugation on sucrose [7,22,5].

The HBc core particles purification strategy we applied on the crude extracts was inspired by this review and adapted according to the constructions, the hosts and a purity–yield compromise in line with our objectives.

Therefore, the HBc core particles expressed by *E. coli* E3CB was extracted according to the particle size and macromolecular behaviour, by using diafiltration on an M_r 300 000 cut-off membrane. However even with extensive buffer dilution, the washing out of contaminant proteins remains rather poor (14%). Despite optimised transmembrane pressure, recirculation and permeate flow-rates, the pore size membrane was probably reduced by a protein polarisation layer at the membrane surface [26] which limited the elimination of host proteins. These observations were also established [27] during the downstream processing of HIV-1 core and virus-like-particles and we can share the authors opinion that ultrafiltration is a well designed method for virus concentration with high yield, but results in a poor purification factor.

The histidine tag construction performed with *E. coli* pOL032 led to the use of Ni-NTA chromatographic gel. Contrary to a similar approach [13], we chose to denature the HBc core particles by lysing the cells in the presence of urea in the lysis buffer to enable the accessibility of the histidine C-terminal tag. Metal chelate affinity chromatography adsorption and imidazole elution was run under these conditions. Refolding and polymerisation of HBc core particles was performed by overnight dialysis in a buffer containing a widely applied redox couple of reduced and oxidised glutathione in the presence of 0.5 *M* L-arginine, as described [28].

We also tried renaturation on the affinity gel by decreasing the urea concentration (gradient) before the imidazole elution, but we obtained similar results (data not shown). The efficiency of metal affinity chromatography to extract histidine tagged protein from crude extract is shown by recovering 86 mg of total protein in the elution fraction from 30 g cells wet mass.

As regards the HBc core purification from *P. pastoris* lysate, the double precipitation technique [25] was applied. Ammonium sulfate precipitation from 30 to 50% has long been used to isolate or concentrate the HBc core from *E. coli* extract. As some contaminants remain, their precipitation occurred under the 65°C heat treatment while the HBc core particles remain in the supernatant. A lower yield was observed when applying this protocol to the yeast extract, but the lack of a complete lysis of yeast and the level of expression must be considered. It can be underlined that the heat precipitation step has to be run with a low contaminant level, otherwise, the occurrence of coagulated materials could entrap the free HBc particles and thereby decrease the yield of this step.

At this stage, the choice of a lysis protocol and a first extraction step has not only direct influence on the antigen enrichment but also influence the success of the further purification step.

To compare the performance of ultracentrifugation to size-exclusion chromatography on the same basis, the same amounts of prepurified material were submitted to both these purification techniques.

As can be expected on the gel filtration support, the HBc core particles have been eluted at the column void volume as large macromolecules. The chromatographic profile of each lysis extract show the proportion of the retained contaminants on the column, and correlate with the efficiency of the first extraction step. The metal affinity purified fraction of the *E. coli* pOL032 fraction and double precipitation protocol of the *P. pastoris* yBW063 fraction enables a better separation than ultrafiltrate *E. coli* E3CB extract. The direct EIA technique revealed that the antigenic activity is related to the UV profile of the first peak. Electron microscope observations confirm that HBc has been eluted as spherical particles and not as unfolded aggregates. Most of the first purification assays on viruses have been performed on agarose matrix such as Sepharose 2B [11] or dextran matrix Sephadex G 200 [29,30] and since, many types of enveloped and non-enveloped viruses were purified from concentrated cell supernatant by this purification technique.

As expected, ultracentrifugation on a sucrose gradient separated the HBc particles between the 40 and 50% sucrose concentration, as in previous working [5,22]. The fractions, checked by direct EIA technique, are separated similarly for the three HBc constructions. Electron microscope imaging ascertains the presence of HBc core particles, and moreover, it can be observed that there are some particle

aggregates (more than in the homologous particles separated by size-exclusion chromatography). When elaborating a purification process, the ultracentrifugation has often been compared to size-exclusion chromatography: for rubella virus on Sepharose 2B [31], for α-viruses on Sephacryl S 1000 [32], for HIV-1 core and virus-like particles on Superdex 200HR [27]. These authors insisted on the good recovery obtained, and moreover viral integrity and infectivity was better conserved when compared to ultracentrifugation. The choice of the gel separation range were generally adapted to separate contaminants with closed chromatographic behaviour. The Superdex 200 gel performed this separation satisfactorily for the three HBc constructions. SDS–PAGE (Fig. 5) and analytical size-exclusion chromatography (Fig. 4) show the greatest purity for the HBc core particles separated by ultracentrifugation, but with a very low yield. Size-exclusion chromatography allows a higher yield but some contaminants are coeluted. Nevertheless, some low-molecular-mass components can be noticed on the analytical size-exclusion chromatography (Fig. 4) despite the high purity shown on SDS–PAGE analysis (Fig. 5) for the HBc particles separated by ultracentrifugation. We can suggest that the density gradient centrifugation could induce deformation and aggregation of virus-like particles, due to over concentration or dehydration of particles in high sucrose density, and then could produce protein degradation.

Among the many investigations on the subject of HBc, only two references reported precise data on HBc core particles production by genetically modified organisms. A truncated form of HBc antigen missing 39 residues from the C-terminus was expressed in *E. coli* [22] and 90 mg of purified protein from 150 g cells wet mass (i.e., 0.6 mg/g wet mass) were obtained; the primary sequence of HBc antigen was expressed in *E. coli* [25] and the two precipitation protocols yielded were 2.4 mg from 5 g cells wet mass (i.e., 0.48 mg/g wet mass). These results are similar to those obtained with the *E. coli* expressed sequence and the size-exclusion chromatography final purification (0.42 mg/g wet mass for pOL032 and 2 mg/g wet mass for E3CB). The quantities obtained by ultracentrifugation are about fivefold less for the three purified fractions. The level of productivity reached with the *P. pastoris* expressed sequence is the lowest for both purification techniques. This could be explained by the lower level of recombinant protein expression in eucaryotic cells compared to procaryotic cells. However the fermentation of this strain was made at laboratory scale on flasks; a fed-batch fermentation mode in a bioreactor with constant feed of methanol and an improved lysis step would increase the productivity of this protein [33].

The three HBc fractions purified as described above have been used for serological immunoassay. Regarding purity and antigenic activity, the recombinant HBc antigen expressed in *P. pastoris* is the best adapted for the HBc diagnosis requirement. Moreover, better yield was obtained with size-exclusion chromatography.

The main advantages of size-exclusion chromatography are: an easy to use, low cost and scaleable technique, compared with the tedious and meticulous preparation required for ultracentrifugation. However, the drawback of size-exclusion chromatography remains the peak volume which must be reduced by concentration.

In conclusion, this comparison enables us to appreciate the necessity of an adapted strategy from the first lysis step to the polishing step. Total lysis is suitable if a very selective extraction step can isolate the protein of interest. When designing a downstream processing strategy for viruses or virus-like particles purification, the size-exclusion chromatography is well adapted as a non specific method. Moreover, the use of soft eluent conditions enables the isolation of intact particles.

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